REDUCTIVE METHYLATION OF IF-3 AND EFTu WITH [^{14}C]FORMALDEHYDE AND SODIUM CYANOBOROHYDRIDE

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1. Introduction

The extent of interaction of a protein with a receptor is most easily determined when the protein is highly radioactive. It is often easiest to purify the nonradioactive protein and subsequently post-label by appropriate modification. The most commonly employed procedures are iodination of tyrosyl residues, which often leads to inactivation, and reductive alkylation with [^{14}C]formaldehyde and sodium borohydride as described [1].

We found, however, that reductive methylation of initiation factor-3 (IF-3) and elongation factor Tu(EFTu) with $NaBH_4$ at the required high pH yielded proteins of low specific radioactivity (3000–6000 cpm/μg). We therefore explored the use of a milder reductant, sodium cyanoborohydride, that could be employed at lower pH values. Reductive ^{14}C-methylation of IF-3 and EFTu with $NaBH_3CN$ gave specific activities >60 000 cpm/μg protein. The advantages of this procedure and the effect of extensive methylation on the function of IF-3 and EFTu is presented.

2. Materials and methods

2.1. *Materials*

Sodium cyanoborohydride was purchased from Aldrich and [^{14}C]formaldehyde (42 mCi/mmol) was obtained from New England Nuclear. IF-3 was prepared according to [2] and EFTu · GDP according to [3].

2.2. *Reductive methylation of IF-3 and EFTu*

Reductive methylation of IF-3 was performed in a buffer containing 10 mM potassium phosphate (pH 7.5), 0.1 mM EDTA, 7 mM 2-mercaptoethanol, 5% glycerol and 800 mM KCl. Protein concentrations ranged from 1.0–1.8 mg/ml and methylations were successfully carried out with as little as 9.5 μg protein. Equimolar amounts of 0.25% [^{14}C]formaldehyde (42 mCi/mmol) and 1.0% $NaBH_3CN$ were added at a 39-fold molar excess over IF-3 unless otherwise indicated. Mixtures were incubated at room temperature for 60 min, then extensively dialyzed against 5 changes of 250 ml 20 mM Tris (pH 7.6), 1 mM EDTA, 5% glycerol, 500 mM NH_4Cl.

The reaction with EFTu · GDP was carried out in 100 mM sodium borate (pH 9.0), 200 mM KCl, 10 mM $MgCl_2$, 20 μM GDP and 2 mM 2-mercaptoethanol. [^{14}C]Formaldehyde and $NaBH_3CN$ were added as above, but at an ~87-fold molar excess. The final protein concentration was 2 mg/ml. After incubation at room temperature for 60 min, the mixture was treated as above. The resultant protein had spec. act. 59 500 cpm/μg.

2.3. *SDS–polyacrylamide gel electrophoresis*

Gels (0.5 × 9 cm) were 16% acrylamide, 1% *N,N'*-diallyltartardiamide, 0.2 M glycine (pH 8.6), 0.1% SDS, 0.5 M urea, 0.005 M EDTA, 0.05% TEMED and 0.12% ammonium persulfate. Stacking gels (0.5 cm) consisted of 4% acrylamide, 0.4% DAT, 0.25 M sodium phosphate (pH 6.6), 0.1% SDS, 0.5 M urea, 0.005 M EDTA, 0.1% TEMED and 0.2% ammonium persulfate. Electrophoresis was performed at 2 mA/gel for 2 h followed by 6 mA/gel for 2.5–3 h in a tank buffer of 0.2 M glycine (pH 8.6), 0.1%

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SDS, 0.5 M urea and 0.005 M EDTA. The gels were frozen, sliced into 1.0 mm thick slices and counted after room temperature incubation in 5 ml 3% Protosol, 0.4% Omnifluor (NEN) in toluene.

3. Results

3.1. *Reductive methylation of IF-3*

Our initial attempts at reductive methylation of IF-3 were carried out according to [1], using [^{14}C]-formaldehyde and $NaBH_4$. The maximum labeling achieved was 3300 cpm/μg protein. When $NaBH_3CN$ was substituted as the reducing agent, the resultant IF-3 had spec. act. >100 000 cpm/μg. IF-3 which is extensively methylated (>1 methyl group/lysine), however, was found to be deficient in binding to 30 S subunits. Only 3–6% of the recovered IF-3 comigrated with 30 S subunits on sucrose gradients with 25–35% of the radioactivity present as a more slowly migrating shoulder (data not shown). This suggested that the IF-3 has been bound to the ribosomes but that the interaction was not strong enough to withstand the pressure of centrifugation.

Therefore we explored various parameters which would allow us to control the extent of methylation. The most efficient method proved to be variation of the concentration of [^{14}C]formaldehyde added as shown in fig.1. The extent of methylation exhibited a linear dependence on formaldehyde concentration up to a ratio of CH_2O/IF-3 = 140. Varying the $CH_2O/NaBH_3CN$ ratio from 0.28–1.0 resulted in only a small increase in methylation. Therefore, most of our subsequent methylations were performed with equimolar amounts of CH_2O and $NaBH_3CN$, both of which were present at a 39-fold molar excess over IF-3. This yielded protein with spec. act. 40 000–60 000 cpm/μg.

Analysis of the labeled protein by SDS–polyacrylamide gel electrophoresis (fig.2) showed that >90% of the radioactivity migrated as a 22 500 mol. wt protein, the expected mobility of intact IF-3 [2]. This indicates that the modification procedure did not result in any appreciable degradation of the native IF-3.

Such reductively methylated [^{14}C]IF-3 retained its activity in two functional assays. Binding to 30 S subunits, as measured by sucrose density gradient

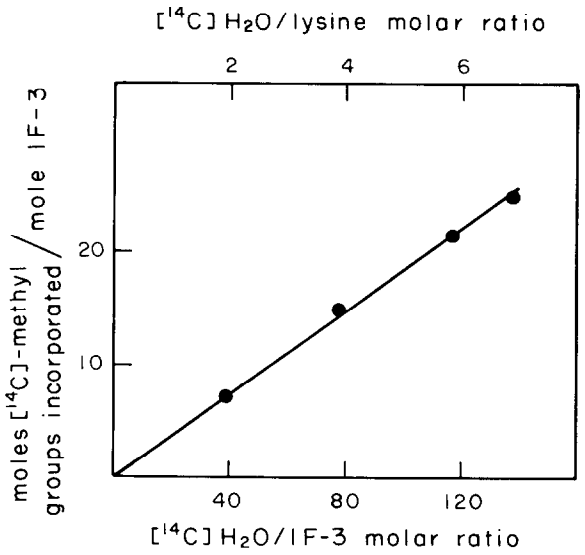

Fig.1. Effect of formaldehyde concentration on the reductive methylation of IF-3. Samples contained 9.5 μg IF-3. 0.1% [^{14}C]formaldehyde (42 mCi/mmol) was added to yield the indicated molar ratios to IF-3. $NaBH_3CN$ was present in equimolar amounts with formaldehyde. After 60 min at room temperature, protein was precipitated with trichloroacetic acid, filtered on glass fiber filters and counted. A blank lacking IF-3 was subtracted from each determination.

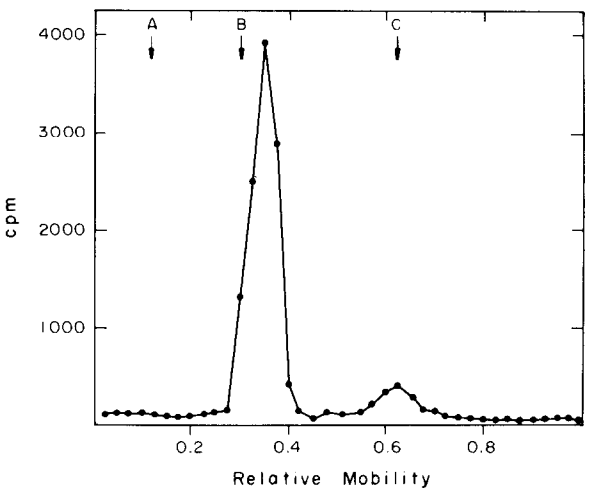

Fig.2. SDS–PAGE analysis of reductively methylated IF-3. [^{14}C]IF-3 0.32 μg (61 300 cpm/μg) was electrophoresed and analyzed as in section 2. Arrows indicated the mobility of marker proteins: (A) ovalbumin (mol. wt 43 000); (B) α-chymotrypsinogen (mol. wt 25 000); (C) cytochrome *c* (mol. wt 12 600).

Table 1
Stimulation of f-met-tRNA binding to 70 S ribosomes by modified IF-3

Additions	cpm bound	% stimulation
–IF-3	4418	–
+ unmodified IF-3 (2.77 μg)	11 790	2.67
+ [^{14}C]IF-3 (0.83 μg)	7404	1.68
+ [^{14}C]IF-3 (1.65 μg)	9187	2.08
+ [^{14}C]IF-3 (3.3 μg)	11 891	2.69

f-met-tRNA binding to 70 S subunits was performed in a 100 μl mixture containing 50 mM Hepes (pH 7.5), 130 mM NH_4Cl, 5 mM $Mg(OAc)_2$, 1 mM dithiothreitol, 0.5 mM GTP, 5 μg poly(A,U,G), 0.7 A_{260} units of 70 S ribosomes, 7.5 pmol/f-[^{3}H]met-tRNA (6600 cpm/pmol), 0.6 μg IF-2 (70% pure) and varying amounts of IF-3 (0.8–4.0 μg). The mixture, lacking tRNA and the initiation factors, was preincubated at 37°C for 15 min to activate the ribosomes [4] and, after addition of the other components, incubation was continued for 15 min at 25°C and the binding measured by adsorption to nitrocellulose filters

centrifugation, was normal. Of the input IF-3 30–40% was bound to 30 S subunits when both were present at equimolar concentrations and this binding was saturable, leveling off at 1.07 mol IF-3 bound/mol 30 S subunits at an IF-3/30 S input ratio of 2.5 (data not shown). This modified IF-3 was nearly as active as control, unmethylated IF-3 in stimulating the binding of f-met-tRNA to 70 S ribosomes in the presence of IF-2 (table 1).

3.2. *Reductive methylation of EFTu · GDP*

A similar methylation procedure as described for IF-3 was applied to EFTu · GDP except that in this case the pH was 9.0 (the EFTu was to be used in subsequent assays which required pH 9). Once again, much higher labeling was achieved in the presence of $NaBH_3CN$ as the reducing agent, 60 000 cpm/μg as compared to 6000 cpm/μg with $NaBH_4$. The methylated EFTu is highly active in stimulating enzymatic binding of the phe-tRNA to 70 S ribosomes (fig.3).

4. Discussion

Both IF-3 and EFTu have been labeled before with [^{14}C]formaldehyde and $NaBH_4$ [5–9]. In [7] the maximum labeling of IF-3 was 21 150 cpm/μg.

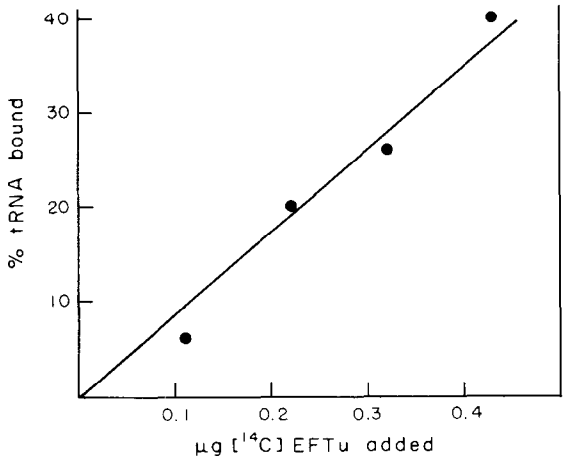

Fig.3. Stimulation of phe-tRNA binding to the ribosomal A site by [^{14}C]EFTu. EFTu-dependent binding of phe-tRNA to ribosomes was measured in a 100 μl mixture containing 2.5 pmol [^{3}H]phe-tRNA, 0.9 A_{260} 70 S ribosomes, 5 μg poly(U) and EFTu · GDP as indicated in 50 mM Hepes (pH 7.4), 75 mM NH_4Cl, 75 mM KCl, 8 mM $MgCl_2$, 1 mM dithiothreitol, and 0.5 mM GTP. Ribosomes were activated by preincubation at 37°C for 15 min before the addition of EFTu to tRNA. 0.56 A_{260} units of crude tRNA were added prior to the phe-tRNA in order to block EFTu-independent binding. Mixtures were then incubated at 30°C for 25 min and the binding measured by adsorption to nitrocellulose filters.

This can be increased at least 3-fold by the procedure described here without affecting the function of IF-3. Such IF-3 contains 16 mol methyl groups/mol protein. Reductive alkylation with formaldehyde and $NaBH_4$ favors dimethyllysine formation [10] but at low excess of $NaBH_3CN$ to amine the subsequent reaction with a second carbonyl group is not favored [11]. Therefore, it is not clear whether the reaction described represents the modification of 8 lysine residues 16 lysine residues or some intermediate number.

It is interesting to note that IF-3 labeled above 100 000 cpm/μg had altered affinity for the 30 S subunit. Since the pK of mono- and di-methyllysine is only slightly altered from that of lysine [10] the modification should not affect any electrostatic interaction between IF-3 and the ribosome. Similarly, because of the small size of the methyl group it does not seem likely that steric hindrance of the interaction occurred. It is thus tempting to speculate that extensive modification interferes with a hydrogen

bonding interaction between some lysines in IF-3 and appropriate acceptors on the ribosome.

EFTu also retained its functional activity after extensive modification. The extent of ^{14}C-labeling described here represents a 4-fold increase over that obtained in [9]. EFTu at 59 500 cpm/μg has 29.5 mol methyl group/mol protein and this represents 74% of the maximum possible labeling (assuming complete dimethylation of all lysines; EFTu contains 20 Lys/molecule [12]). It was shown [13] that reductively methylated EFTu (using formaldehyde and $NaB[^3H]_4$) was active in stimulating polyphenylalanine synthesis and we confirm that its ability to stimulate binding of aa-tRNA to the A site is unaltered.

$NaBH_3CN$ has been used in the reductive methylation of proteins. It was found [14] that the reductive alkylation of BSA at pH 7.2 showed a linear dependence on the aldehyde concentration (in this case pyridoxal) but that a similar variation in the $NaBH_3CN$ concentration had little effect. We have confirmed this behavior in the case of IF-3 alkylation (fig.1). This is to be expected based on the proposed mechanism of the reaction [10]. Since formation of the Schiff's base is the slow step, an increase in the aldehyde concentration would be expected to result in greater modification. Reduction, on the other hand, occurs very rapidly and should only require stoichiometric amounts of $NaBH_3CN$. The reductive methylation of a series of proteins with $[^{14}C]CH_2O$ and $NaBH_3CN$ was reported [15] but no data on the subsequent functional activity of such extensively methylated proteins was presented. The experiments presented here not only confirm the labeling technique but demonstrate that the activity of the modified proteins can be maintained.

The substitution of $NaBH_3CN$ for $NaBH_4$ as the reducing agent has a number of advantages.

(1) The reaction can be performed at neutral pH, rather than pH 9 or above. This is so because $NaBH_3CN$ is stable down to pH 3–4 [11] unlike $NaBH_4$ which is unstable at neutral pH. Since formation of Schiff's base is maximal at pH 6 (albeit unfavored) [11] a lower pH enhances the extent of methylation.

(2) Smaller amounts of CH_2O are required for the methylation. This is again a reflection of the greater stability of formaldehyde at neutral pH. Our conditions for the methylation of IF-3 (to 60 000 cpm/μg) required only a 2-fold molar excess of CH_2O over lysine as opposed to that in [1] which uses a 5-fold excess and only results in protein with, at best, 33% of the specific radioactivity.

(3) Much higher levels of labeling can be achieved with $NaBH_3CN$ and this procedure can be applied to a wide variety of proteins without any major effect on function.

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